UNIVERSITY OF GHANA

COLLEGE OF HEALTH SCIENCES

PHENOTYPIC AND GENOTYPIC ANALYSIS OF *ONCHOCERCA VOLVULUS* RESPONSE TO IVERMECTIN TREATMENT

JUDITH ESINAM YABANI

DEPARTMENT OF MEDICAL MICROBIOLOGY

SCHOOL OF BIOMEDICAL AND ALLIED HEALTH SCIENCES

JULY, 2017

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BY

YABANI, JUDITH ESINAM

(10507341)

THIS THESIS IS SUBMITTED TO THE UNIVERSITY OF GHANA, LEGON IN PARTIAL FULFILMENT OF THE REQUIREMENT FOR THE AWARD OF MASTER OF PHILOSOPHY DEGREE IN MEDICAL MICROBIOLOGY

DEPARTMENT OF MEDICAL MICROBIOLOGY

SCHOOL OF BIOMEDICAL AND ALLIED HEALTH SCIENCES

JULY, 2017

DECLARATION

DEDICATION

This work is dedicated to the Almighty God, my parents and siblings.

ACKNOWLEDGEMENT

This work would not have been possible without the grace of God; therefore my utmost thanks go to Him for his strength, knowledge and protection throughout my three years of academic work.

I am very grateful to my supervisors, Dr. Simon K. Attah and Rev. Prof. Patrick F. Ayeh-Kumi for their immense supervision and guidance for the successful completion of this research. I wish to express my heartfelt gratitude to Dr. Mike Osei-Atweneboana of the Water Research Institute (WRI), the Council for Scientific and Industrial Research Institute (CSIR) for his guidance, unflinching support, and for making this project a reality by providing the necessary logistics from his laboratory and research grant for the entire work.

I am thankful to the entire staff of the Biomedical and Public Health Research Unit of the Water Research Institute, CSIR for their assistance and encouragements in making this research a success.

My unreserved appreciations go to the lecturers, technicians and secretaries of the Medical Microbiology Department, School of Biomedical and Allied Health Sciences for the support they provided in the successful completion of this work.

Lastly, my gratitude and sincere thanks go to my parents, Mr. and Mrs. E.K.G Yabani and my siblings for their unceasing encouragement, spiritual and financial support.

ABSTRACT

Ivermectin remains the only potent drug for the control and mass treatment of onchocerciasis. Nonetheless, recent studies indicate sub-optimal responses and genetic changes in some populations of the adult *Onchocerca volvulus*. Hence, more studies are required to determine whether resistance is developing. This requires analysis of phenotypic and genotypic responses of *O. volvulus* to ivermectin treatment to determine the association between worm phenotype and genotype after treatment. Forty archived *O. volvulus* female worms were obtained from patients hailing from three Ghanaian endemic communities: Asubende, Kyingakrom and Agborlekame. These communities were known to harbour both good and poor ivermectin response groups. The female worms were retrieved from nodules removed from patients who previously had undergone 16-17 rounds of (150 µg/kg) ivermectin treatment with one year interval between treatments. The samples were obtained 3 months after the last round of treatment. Genetic analysis of the beta-tubulin gene of the adult worms and their pooled microfilariae (MF) on a 538 bp DNA fragment of the beta-tubulin gene as well as restriction was done to determine mutations in the beta-tubulin gene. It was detected that 70% (28/40) of the worms harboured greater than 70% of normal stretched MF in their genital tracts; these were considered as poor responders to ivermectin treatment. On the other hand, 30% (12/40) of the worms that harboured less than 10% of the stretched MF in their genital tracts were considered as good responders to ivermectin treatment. Molecular analysis indicated single nucleotide polymorphism (SNP) for both adult worm and their corresponding MF with the heterozygote mutant adult worms showing strong association with the MF. In conclusion, it is found in this study that some of the worms have responded poorly or sub-optimally to ivermectin treatment. This development stems from

mutations in the beta-tubulin gene of the adult female worms that have been passed on to their progenies.

LIST OF ABBREVIATIONS

ATP	Adenosine Triphosphate
APOC	African Programme for Onchocerciasis Control
CDC	Centers for Disease Control and Prevention
CDTi	Community Directed Treatment with Ivermectin
DALY	Disability-Adjusted Life Years
DEC	Diethylcarbamazine
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide Triphosphate
GDP	Guanosine Di-Phosphate
GTP	Guanosine Tri-Phosphate
L1s	First stage Larvae of *O. volvulus*
L3s	Third stage Larvae of *O. volvulus*
MDA	Mass Drug Administration
MF	Microfilariae
ML	Macrocyclic lactone
OCP	Onchocerciasis Control Programme
OEPA	Onchocerciasis Elimination Programme for the Americas
OSD	Onchocerciasis Skin Disease
PCR	Polymerase Chain Reaction
Pgp	Permeability glycoprotein
PM	Peritrophic matrix
RFLP	Restriction Fragment Length Polymorphism

RPM	Revolution per minute
SNP	Single Nucleotide Polymorphism
SOR	Sub-Optimal Response
UV	Ultra-violet
WHO	World Health Organization

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CHAPTER ONE

1.0 INTRODUCTION

1.1 General Introduction

Onchocerciasis or "river blindness'' is an infectious disease caused by the parasitic filarial nematode *Onchocerca volvulus*. This parasite exists in two main forms, the adult (male or female) worms and the skin-dwelling embryonic larvae called microfilariae (MF). The disease is a global burden and geographically, it is distributed in Africa, South and Central America and Yemen (MMWR, 2013). It is estimated that 37 million people are infected globally with the parasite out of which 99% live in Africa. Over 90 million people are at risk of acquiring the infection in 29 Sub-Sahara African countries. Farmers, hunters, fishermen and people who reside close to *Simulium* breeding sites are the risk population. Studies have shown that adult males are highly infected than females and children (CDC, 2013).

The third stage infective larvae (L3s) of the parasite are transmitted into the skin of the human host when an infected *Simulium* fly bites to ingest blood. The L3s penetrate the bite wound and develop into adult males and females in the subcutaneous tissue where they mate and live up to about fifteen years (or an average of twelve years). The gravid female worm produces about 1,000 MF daily. These MF which can live for two years are responsible for most of the morbidity associated with the disease (CDC, 2013).

Among infection related diseases that result in blindness, onchocerciasis is rated as the second leading cause of this disability after trachoma. It is also estimated that onchocercal skin disease (OSD) which causes troublesome itching accounts for 60% of healthy life-years lost annually due to the disability and mortality associated with the disease (Remme *et al*., 2002). Other effects of *O. volvulus* infection are epilepsy due to heavy infection, social ostracism,

troublesome itching associated with disfiguring skin lesions which makes it difficult for one to work, study and interact socially, reduced life expectancy rate among infected individuals with high MF load, high mortality rate among the blind and reduced socioeconomic status of affected communities as a result of low productivity and abandonment of fertile river valleys (Prost 1986; Vlassof *et al*., 2000; Murdoch *et al*., 2002; Little *et al*., 2004; Pion *et al*., 2009). Other negative impacts associated with the infected individuals are increased health expenditure and diminishing income generating capacity (Pion *et al*., 2002).

Ocular onchocerciasis is diagnosed through an ophthalmological examination of the visual function of the eye, presence of MF in the intraocular eye and other pathological changes related to the infection. Microscopy is used to detect the MF from skin biopsies, whiles the adult worms are detected after surgical removal of nodules from the body followed by their digestion with collagenase to expose the adult worms or by histological processing of the nodules (WHO, 1995). According to Zimmerman *et al*. (1994), DNA probes through polymerase chain reaction (PCR) have been developed to determine the presence of the *Onchocerca* parasite in routine skin snips.

In order to control the disease, some strategies were developed and implemented. These include the establishment of the Onchocerciasis Control Programme (OCP), African Programme for Onchocerciasis Control (APOC) and the Onchocerciasis Elimination Programme for the Americas (OEPA). These programmes were tasked to control the disease by reducing its burden to a level where it will no more be of public health importance.

According to the previous studies, ivermectin is the only safe drug recommended for the treatment and control of the *O. volvulus* infection and the only drug proven to be safe for the treatment of the disease (WHO, 2017).

1.2 Problem Statement

There are reports of sub-optimal response (SOR) of *O. volvulus* to ivermectin treatment and this has been associated with mutations in the beta-tubulin gene of some female worms. Reports indicate that there is SOR in some endemic communities in Ghana (Awadzi *et al*., 2004; Osei-Atweneboana *et al*., 2007; Basáñez *et al*., 2008; Pion *et al*., 2013). It is not clear if the worms from the study sites have mutations in the beta-tubulin gene. Confirmation is required for the findings from these studies and the current study was carried out to do that.

The apparent emergence of resistance of *O. volvulus* worms in some endemic communities to ivermectin may be attributed to the prolonged reliance of ivermectin for mass drug administration (Taylor *et al.*, 2009). This situation is critical to the control of *O. volvulus* and poses a significant threat to the fight against onchocerciasis and a high probability of disruption of the success of the onchocerciasis control programme (OCP) and the African programme for onchocerciasis control (APOC). It is therefore imperative to ascertain if there is any emerging resistance in the endemic communities where the samples were collected from for this study.

1.3 Justification

Ivermectin is the main drug being used for onchocerciasis control. Therefore, the development of resistance in the MF will throw the control of onchocerciasis out of gear. Early detection of resistance in the progenies of the adult worm will enable the deployment and integration of new management tools in the fight against the disease.

1.4 Aim

The aim of this study was to analyze the phenotypic and genotypic responses of *O. volvulus* to ivermectin treatment.

1.5 Specific Objectives

The specific objectives of the study were to determine:

- the effect of ivermectin on the adult female worms and their progenies particularly, the intrauterine MF,
- if the genetic changes in the beta-tubulin of the female *O. volvulus* is associated with SOR to ivermectin treatment, and
- if these genetic changes in the beta-tubulin have been passed on to the progenies of the female worm.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 The Parasite

Onchocerca volvulus is a filarial parasitic worm that belongs to the Superfamily Filarioidea. It is an obligate parasite that causes the disease, onchocerciasis. It exists as adult (macrofilariae) or microfilariae (Udall, 2007). The macrofilariae are found in the subcutaneous tissues entangled in painless, firm and usually palpable nodules called onchocercomata. The MF is found mainly in the skin although they can be found in other body specimens such as blood, urine and sputum when their numbers are high in the body (CDC, 2013).

2.2 Morphology of *Onchocerca volvulus*

2.2.1 Macrofilariae

The macrofilariae comprise both adult male and female worms that are normally intertwined in the nodule. Both sexes are whitish in colour which changes to brown with maturity. Each worm has a blunt head, and a tapering posterior end or tail. The females are larger in size than the males and measure 33 to 50 cm by 270 to 400 µm with narrow anterior ends. The reproductive system of the females consists of paired ovaries, oviducts, seminal receptacles and uteri. Male worms measure 19 to 42 mm by 130 to 210 µm. They have a reproductive system that consists of a straight tube which runs through them (CDC, 2013).

Figure 2.1: Adult males and females entwine; Source: Schulz-Key, 1988.

2.2.2 Microfilariae

The MF measure 220 to 360 µm by 5 to 9 µm and is unsheathed. They have a blunt anterior portion and a tapering posterior end as shown in figure 2.2 below.

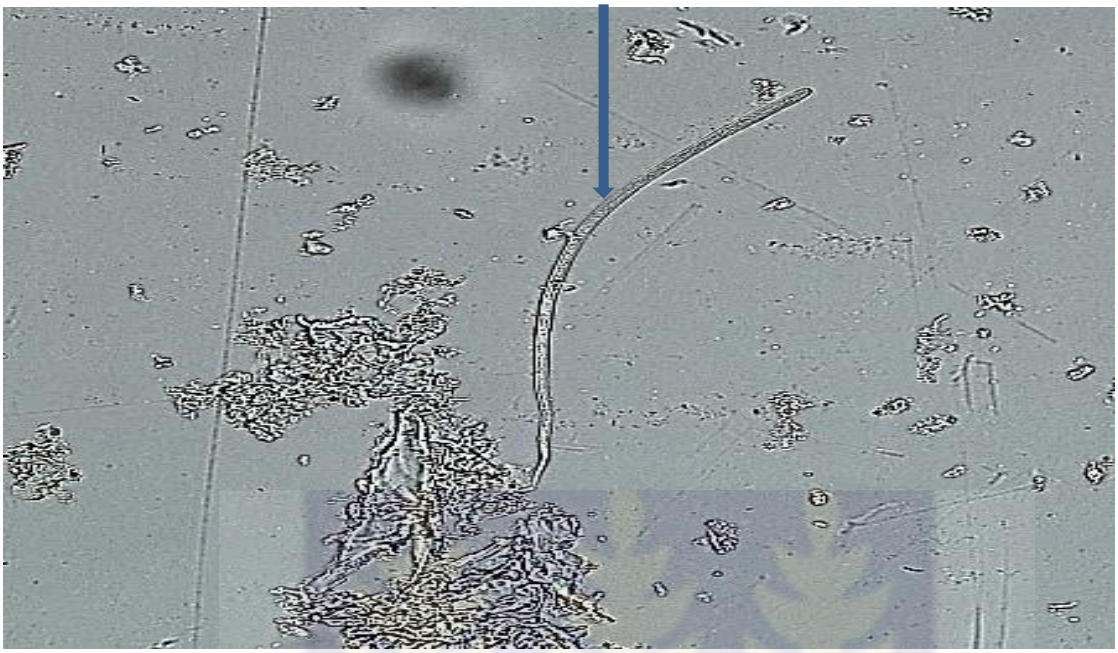

Figure 2.2: Microfilaria of *Onchocerca volvulus* (arrow); Source: Original

2.3 Life cycle of *Onchocerca volvulus*

The parasite has an indirect life cycle in which the blackfly (vector) serves as the intermediate host and humans serve as the definite host. An individual becomes infected when the L3s of the parasite are inoculated into the skin of humans after the blackfly takes a blood meal. The L3s, after two years, develop into adult worms which permanently reside in the subcutaneous tissues and live for almost 15 years. The adult male and female then mate and produce several MF that move to the skin, eyes and other organs of the body. The female worms reside in the nodules and produce the MF for approximately 9 years. The MF has a life span of two years (CDC, 2013).

The transmission cycle continues when the female blackfly ingests the MF in the course of taking another blood meal. The blackfly ingests several MF during a single bite most of which are engulfed and digested in the peritrophic matrix (PM) secreted by the fly midgut. Only a few

MF penetrate the PM into the stomach wall and further invade the epithelial layer of the stomach into the abdominal and thoracic cavities (CDC, 2013). The MF develops into the sausage-like first stage larvae (L1s) and later develops into L3s which measures up to 650 µm in length. The L3s then migrate to the blackfly's proboscis to infect another human when the fly ingests a blood meal.

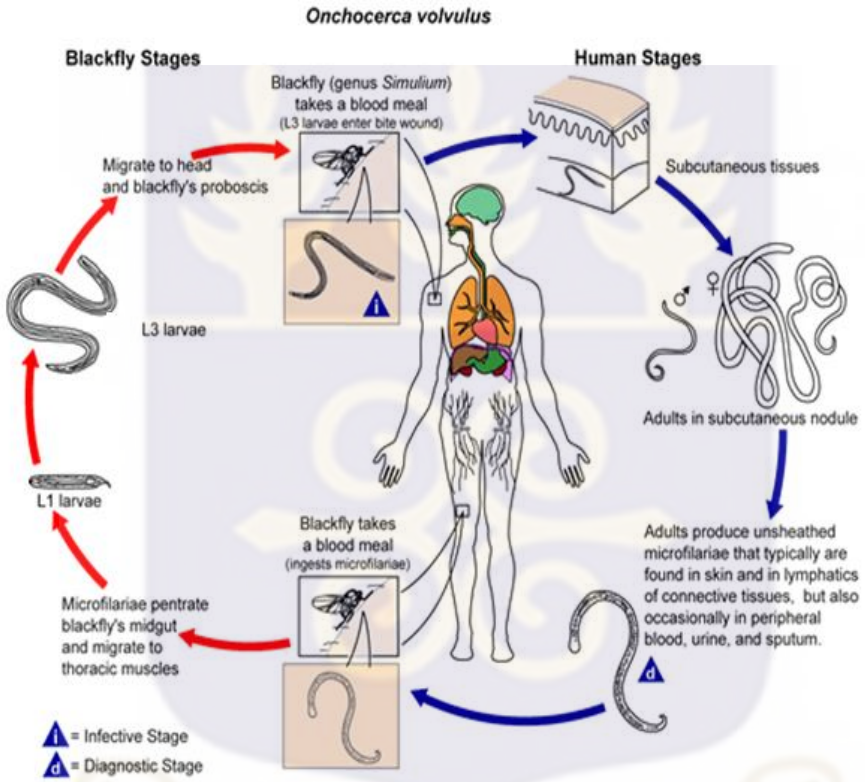

Figure 2.3: Life cycle of *O. volvulus* (Accessed from: http://www.cdc.gov)

2.4 The disease

2.4.1 Clinical presentations

The MF are the primary cause for the morbidity accompanied with the disease. When the MF die, they cause intense inflammatory reactions in the skin leading to skin rashes, lesions, and

intense itching (pruritis). Inflammatory lesions or onchodermatitis appear as small, round red bumps on the skin. Prolonged scratching of the affected parts destroys the elastic fibres of the skin and causes the skin to appear thin and wrinkled, a condition referred to as like a lizard skin. Skin depigmentation in the affected area can be found in later stages of the infection and it is known as leopard skin. In other conditions, the skin may become thickened and it is referred to as elephant skin. Another uncommon complication associated with onchocerciasis is lymphadenitis which sometimes leads to hanging groins and elephantiasis of the genitals as well as epilepsy and hyposexual dwarfism. In persons with worm burden, the MF migrates to the eyes, die and release an endosymbiotc bacterium known as *Wolbachia*. The death of this bacterium causes the release of toxins which causes inflammatory responses that lead to the beginning of impaired vision and finally blindness (WHO, 1995; Brattig, 2004; Higazi *et al*., 2005).

Some of the effects of *O. volvulus* infection are severe itching, blindness, epilepsy due to heavy infection, high mortality rate in the blind which leads to a short life expectancy rate and abandonment of fertile river valleys (Prost, 1986 ; Pion *et al*., 2002; Little *et al*., 2004).

2.5 Diagnosis

There are different methods of diagnosing onchocerciasis, but the gold standard is the skin snipping. This method involves the use of a sclerocorneal punch, scapel or blade to obtain skin biopsy from the iliac crests, scapular and calves. The skin snips are placed in a 96-well plate containing about 100 µl of physiological saline (pH 7.0) and incubated to allow the MF to emerge from the skin snip over a 24 hour period. The MF is enumerated with the aid of an inverted microscope, or an ordinary light microscope. Skin snip microscopy is not a sensitive

diagnostic tool in areas with low infection prevalence as a result of either low transmission rate or successful ivermectin treatment programmes.

Nodule examination can be done by palpation of affected skin areas but this technique cannot be used to determine infectivity since some nodules are located in deep muscles, with the most active and very small nodules usually very difficult to palpate (Thylefors, 1978; Duke, 1993). Recent biochemical methods such as skin snip PCR, enzyme linked immunosorbent assays (ELISA), enzyme immunosorbent assay (EIAs) and antigen detection have been extensively used to develop more sensitive and reliable means of infection diagnosis (Ngomou, 1994; Alley, 2001). There are other methods such as: ultrasonography, histology and collagenase digestion of nodule, slit lamp microscopy for detecting MF and Mazzotti test (WHO, 1995).

2.6 Socioeconomic consequences

Among people with onchocercal skin disease (OSD), males are faced with challenges related to their ability to achieve economic prospects and their sexual performances whiles females are challenged with the probability of getting married due to their disfigured physical appearances as a result of OSD (Vlassoff *et al*., 2000). Also, people with OSD have low income (Oladepo *et al*., 1997) and they suffer stigmatization which affects them in all facets of their lives (Mbanefo *et al*., 2010).

2.7 Treatment

2.7.1 Diethylcarbamazine

The World Health Organization (WHO) introduced diethylcarbamazine (DEC), a microfilaricide for the treatment of the disease after the Onchocerciasis Control Programme

(OCP) was launched in West Africa with the aim of eliminating the disease. However, it was realized that, treatment of the disease with this drug had intrinsic toxic side effects and could not be used for mass treatment (Francis *et al*., 1985; Greene *et al.*,1985 ; Awadzi and Gilles, 1992).

2.7.2 Suramin

Suramin is a macrofilaricide that was later introduced for the treatment of onchocerciasis but it also has little effect on the MF. It is not safe (Thylefors and Rolland, 1979) so, it is recommended to be administered to patients under the supervision of an experienced physician.

2.7.3 Ivermectin

Ivermectin, a semisynthetic drug with a broad spectrum antiparasitic activity, belongs to the avermectin family. It was isolated from the fermentation products of *Streptomyces avermitilis.* It contains a mixture of 5-*O*-dimethyl-22, 23-dihydroavermectin A1a and 10% 5-*O*-dimethyl-25-de (1-methylpropyl)-22, 23-dihydro-25-(1-methylethyl) avermectin A1a, with molecular weights of 875.10 and 861.07 respectively (Merck & Co, 2007).

This is a potent drug for veterinary animals, which was introduced in 1987 for the treatment of human onchocerciasis. It was soon added as an adjunct to vector control after it was donated free of charge by its manufacturer (Merck & Co, 2007) to the OCP for as long as needed. Ivermectin is not a macrofilaricde. Studies have indicated that administering it annually is very safe, prevents onchocerciasis and onchodermatitis, and has an enormous impact on reducing the transmission of the disease (Greene *et al.,* 1985; Remme, 2004).

Ivermectin is widely used against both endoparasites and ectoparasites. In human medicine, ivermectin is used in dermatology against arthropods such as human head and body lice (Strycharz *et al*., 2008) as well as scabies (Dourmishev *et al*., 2005; Khan & Yasmin, 2007). An analysis has shown that oral ivermectin can be considered as an alternate method of treating severely crustered scabies lesions in immunocompromised patients compared to topical treatment which is expensive and less effective (Fawcett, 2003). A recent study has shown the success of ivermectin as a parenteral veterinary formulation in treating disseminated strongyloidiasis (Marty *et al*., 2005). Additionally, it has been shown that topical ivermectin is of great importance in treating worm larvae infestation (myiasis) (Dourmishev *et al*., 2005) and cutaneous larval migrans (Caumes *et al.,* 1993; Bouchaud *et al*., 2000). Also, ivermectin is used in treating other filarial infections (Bockarie *et al*., 1998; Boussinesq *et al*., 1998 ; TCC, 2013).

2.7.3.1 Pharmacodynamics and mode of action of ivermectin

The avermectin and milbemycin exhibit two basic effects on nematodes; the inability to pump food into the pharynx and paralysis. Ivermectin has high affinity for and binds with glutamate gated chloride ion channels (GluCl) found in the cells and muscles of nematodes. When ivermectin binds with GluCl, it irreversibly activates GluCl receptors which depolarize the pharyngeal muscle leading to a high level of internal chloride ions. The presence of GluCl on the pharyngeal muscle prevents the pharynx from pumping food to the worm and starves it to death (Pemberton, 2001). Secondly, ivermectin inhibits neuromuscular transmission and paralyzes the worm's body wall muscle (Wolstenholme and Rogers, 2005).

2.7.3.2 Contraindications, adverse effects and precautions

Itching, ocular irritation reactions, headache, arthralgia, myalgia, lymphadenopathy, fever and oedema can occur. In patients with co-infection of *Loa loa* and *O. volvulus*, severe reactions such as encephalopathy occur if the MF of *L*oa *loa* exceeds 30,000 MF/ ml, and in case of more than 8,000 MF/ml functional impairment can occur. Thus, the drug is to be administered with caution especially in communities where *L*oa *loa* with *O. volvulus* is co-endemic. Pregnant women and children below 15 kg body weight are excluded in ivermectin treatment since safety is not guaranteed (WHO, 2013).

2.7.4 Doxycycline

One of the several drugs currently under consideration is doxycycline. Reports indicate that doxycycline has a sterilizing effect on the macrofilariae and clears all developmental stages as well as the *Wolbachia* and had no adverse effect on treated subjects (Turner *et al*., 2010).

2.8 Control of Onchocerciasis

2.8.1 Onchocerciasis Control Programme (OCP)

The Onchocerciasis Control Programme (OCP) was launched in West Africa in 1974 as a collaborative initiative of four United Nations (UN) agencies namely: WHO, United Nations Development Programme (UNDP), the World Bank and the Food and Agriculture Organization of the United Nations (FAO). The OCP employed the strategy of spraying the breeding sites of the blackfly vector in fast flowing rivers and streams to kill its larval stages with environmentally friendly insecticides over a period of three decades. The onset of

ivermectin for the treatment of human populations changed the principle of control from the sole reliance on larviciding to a combination of both vector control and parasite control. The OCP successfully controlled the disease as a public health burden in its area of operation. Other achievements of the OCP include the freeing of 18 million children who have at the risk of blindness, the prevention of 600,000 people from becoming blind and reclamation of 250, 000 km^2 of abandoned (Harlem, 2002; Hopkins, 2005).

2.8.2 African Programme for Onchocerciasis Control (APOC)

The African Programme for Onchocerciasis Control (APOC) was launched in 1995 as a sole antiparasitic agency with the main aim of consolidating and extending the OCP gains to 19 other endemic African countries which did not benefit from vector control by the OCP. The APOC adapted the strategy of community directed treatment with ivermectin (CDTi) and was successful in the various communities that it operated within (Seketeli *et al.*, 2002). After a successful significant reduction of infection was observed in communities that had taken the medication for more than 15 years (Borsboom *et al*., 2003), it was predicted that, the disease will not be a public health problem for more than 20 years if the treatment is suspended **(Plaisier *et al.*, 1995).** It was deduced that, if CDTi is used to cover 70% of endemic areas, and 80% of these areas are able to maintain annual treatment at 65% coverage for a period of less than 15 years, 26 million DALYs would be prevented over a period of 25 years.

2.8.3 Onchocerciasis Elimination Programme for the Americas (OEPA)

The Onchocerciasis Elimination Control Programme for the Americas (OEPA) was launched with the intension of eradicating onchocerciasis in the Americas after several years of control

using biannual treatment regimen. The transmission of the disease was interrupted in four of the six endemic countries in the Latin America by the end of 2012. The American programme used bi-annually ivermectin Mass Drug Administration (MDA) coupled with health education and mobilization of affected communities in endemic foci in the six affected countries (Blanks *et al*., 1998; Sauerbrey, 2008). The endemic communities targeted for MDA were classified into three categories namely; hyperendemic, mesoendemic and hypoendemic. After the MDA which was carried out from 1993 to 2012, the OEPA was found to be successful in interrupting transmission in 11 out of 13 foci (CDC, 2013). Transmission was found to have been interrupted in Colombia and Ecuador in 2007 and 2009 respectively and in Mexico and Guatemala in 2011. Colombia, Ecuador and Guatemala were declared free of onchocerciasis in 2013, 2014 and 2015 respectively. Effort to eliminate the disease is now focused on the Yanomami of Brazil and Venezuela (WHO, 2017).

2.9 Onchocerciasis in Ghana

A study report revealed that onchocerciasis is endemic in nine out of the ten regions of Ghana; the exception being the Greater Accra region (Taylor *et al*., 2009). It was realized that, there are 3,204 endemic communities from 66 districts in the 9 regions. Of these 3,204 communities, 247 are hyperendemic areas and have been described as a special intervention zone. The findings from the entomological and epidemiological surveillance activities carried out by the APOC indicated the necessity to improve monitoring for fly infectivity levels and infection in humans. Surveys done in 2005 and 2006 showed high infectivity rates of parous flies from the Pru river basin sites and in 2006 within the White Volta, Kulpawn, Anum and Pra river basins (Taylor *et al*., 2009). In co-endemic communities with lymphatic filariasis and onchocerciasis,

ivermectin treatment were introduced in 2001 and by 2005, 61 endemic communities were covered.

2.10 *Onchocerca volvulus* response to ivermectin treatment

2.10.1 Phenotypic responses

A longitudinal follow-up study was carried out by Klager *et al.* (1996) to assess *O. volvulus* nodules from 77 patients in Sierra Leone in a randomized controlled trial. The patients received either 4 annual or 10 monthly doses of ivermectin for more than 6 years. Nine (9) months after the last ivermectin treatments, excised worms from these patients were examined for changes in morphology, viability and reproducibility. The findings indicated a high reduction in the proportion of live male worms in the nodules, and 90% reduction in reproduction in female worms.

Turner *et al*. (2010), in a double blinded randomized field trial study, administered 200 mg/day of doxycycline only and doxycycline in combination with ivermectin (150 µg/kg) to 150 *Onchocerca* infected patients. Twenty two (22) individuals co-infected with *Loa loa* and *O. volvulus* were given the same treatments. The effects of the medications were determined at 4, 12 and 21 months after treatment. It was realized that at the end of the 12 months, doxycycline and ivermectin treated individuals had lower microfilaridermia compared with doxycycline or ivermectin treated individuals only.

In order to determine emerging ivermectin resistance in *O. volvulus*, Osei-Atweneboana and colleagues (2011) conducted a follow up study on 268 microfilaridermia subjects in 9 Ghanaian communities who were previously administered with 10 to 19 annual doses of ivermectin treatment and an ivermectin naïve community. Skin snips were done 364 days after

the initial ivermectin treatment. Skin snips and nodules were taken 90 days after the second ivermectin treatment, to determine the morphological age and MF contents. It was detected that 90% of the worms were classified as middle aged or older. Additionally, the study results also indicated SOR in some adult female worms (Osei-Atweneboana *et al*., 2011).

Katabarwa *et al*. (2013a) assessed the transmission of onchocerciasis and its effect in three health districts in the Northern part of Cameroon, where mass ivermectin treatment had been going on (on annual basis) for 12 to 17 years. In these two year studies, skin snips were assessed for MF, nodules were palpated and MF in the eye was detected using a slit lamp microscopy and vector flies were dissected for the determination of larva rates. The findings revealed that adult subjects had Mf rates of 4.8%, nodule rates of 13.6% and 5.5% of patients had intra ocular MF. The study also revealed significant evidence of ongoing transmission in one of the health districts with 2.6% prevalence rates for children below 10 years of age.

An onchocerciasis transmission study was carried out in West Cameroon where annual mass ivermectin treatment has been going on for 12 to 17 years by Katabarwa and colleagues (2013b). There was a follow up in 2005, 2006 and 2011 to assess MF load in skin snips and palpation examination for nodules. The *Simulium* vector species were dissected to determine larval infection rate from 2011 to 2012. The study results revealed a reduction in MF prevalence in adults from 68.7% to 11.5%; similarly, nodule prevalence dropped from 65.9% to 12.1%. It was also detected that only 3 out of the 11 districts studied were close to interrupting the transmission. There is evidence that transmission is still on going in two of the three fly infested sites where the flies were collected. In view of this, Katabarwa *et al*. (2013b) recommended that annual mass drug administration with ivermectin should proceed after 15 years, since discontinuing the treatment will lead to the increase in transmission rate.

2.11 Genes associated with ivermectin resistance in humans and helminthes

The high rates at which anthelmintics are used in humans and animals are likely to result in selection for resistance. There is strong evidence that resistance is occurring in ivermectin treatment. Ivermectin resistance in veterinary parasites has been genetically associated with permeability glycoproteins (P-gp) and the beta-tubulin gene (Taylor *et al*., 2009). There is evidence that, ivermectin resistance has occurred in the P-gp gene of the veterinary parasites, *Haemonchus contortus* (Xu *et al*., 1998) and *Caenorhabditis elegans* (James & Davey, 2009). Recent studies have associated ivermectin resistance with the beta-tubulin gene of *O. volvulus* (Eng & Prichard 2005; Eng *et al*., 2006; Bourguinat *et al*., 2007; Osei-Atweneboana *et al*., 2007; Tawiah-Gyan, 2013).

2.12 Tubulin gene and its function in drugs

2.12.1 Beta-tubulin gene and its role in ivermectin resistance

Tubulin is a member of the protein superfamily of the globular protein. There are six families of tubulins in this superfamily known as alpha, beta, gamma, delta, zeta and epsilon. Gamma (γ) plays crucial role in the nucleation and polar orientation of microtubules. The delta (δ) and epsilon (ε) tubulins function in the mitotic spindle during mitosis found in centrioles. Zeta is found in kinetoplastid protozoa. The alpha (α) and beta (β) tubulins polymerize into dynamic microtubules. Both are slightly acidic and have an isoelectric point between 5.2 and 5.8 with a molecular weight of about 50,000 Daltons. Microtubules are components of cytoskeleton and are made of basic structural unit of alpha-beta tubulin heterodimer. Microtubules can grow as long as 50 micrometer and are very dynamic in eukaryotic cells and in some bacteria (Pilhofer

et al., 2011). They play very crucial functions in structural support, cytokinesis, motility and transport of cellular structures around cells.

In the Guanosine triphosphate (GTP) bound state, the α- and β-tubulin dimers bind to GTP and gather at the positive ends of microtubules (Heald & Nogales, 2002). The β-tubulin subunit is exposed on the plus end while the α-tubulin subunit is exposed at the minus end of the microtubule. After the incorporation of the dimer in the microtubule, the GTP bound state of the β-tubulin subunit hydrolyses into Guanosine diphosphate (GDP) through interdimer contacts along the microtubule protofilaments (Heald & Nogales, 2002). The stability of the dimer in the microtubule is influenced by the binding of the β-tubulin to either GTP or GDP. In order for the dimers to assemble in the microtubule, they bind to GTP and to disassemble, they bind to GDP. Thus, the GTP cycle is a requisite for the dynamic instability of the microtubule and is therefore a target for anticancer drugs in humans (Giannakakou & Zhou, 2005).

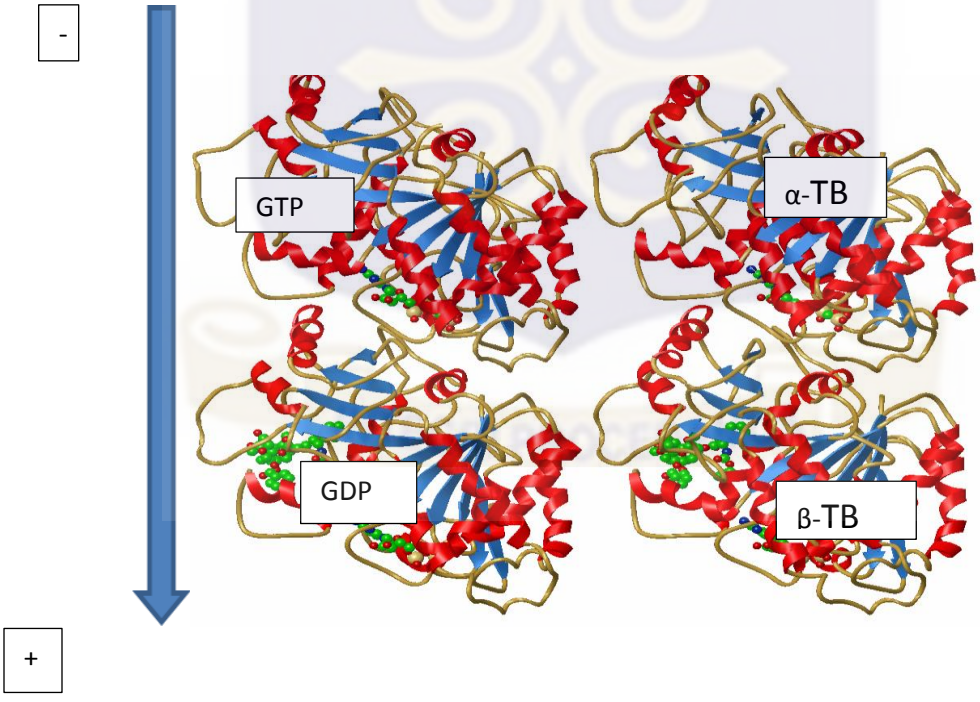

Figure 2.4: Ribbon diagram of the tubulin dimer; Source: Heald & Nogales, 2002.

The figure above illustrates the front view of the microtubule which shows the alpha-tubulin (α-TB) with bound GTP (top) and beta-tubulin (β-TB) containing GDP (bottom). The arrow indicates the direction of the protofilaments and microtubule axis.

The alpha-tubulin is exposed to the minus (-) end whiles the beta-tubulin is exposed to the plus (+) end. The beta-tubulin monomer consists of the drug binding or intermediate domain where ivermectin binds, a nucleotide binding or N-terminal domain and a mitochondrion associated or C-terminal domain. Polymerization of the alpha-beta monomer is prevented from forming microtubules when ivermectin binds to the drug binding domain. This prevents the structural conformation of the drug binding domain to ivermectin. Furthermore, the occurrence of microtubules is prevented, thereby inhibiting the normal functioning of the cell, tissues and organs. On the other hand, mutations in the drug binding domain will alter that domain by inhibiting ivermectin from binding to it and conferring resistance to *O. volvulus*.

2.13 Genetic responses of *Onchocerca volvulus* to ivermectin treatment

In a study by **Huges *et al*. (2012)** to determine whether there is emerging ivermectin resistance in some category of gravid female worms obtained from subjects in hyperendemic communities in Cameroon, the beta-tubulin gene of the worms were assessed for single nucleotide polymorphisms. Samples were collected prior to initial ivermectin treatment and after three years post ivermectin treatments. The study revealed four SNPs in the beta-tubulin gene of the worm. The SNP which occurred at 1545 (A/G) indicated an immense increase in the frequency of female worms after ivermectin treatments.

A twenty one (21) month epidemiological investigation was carried out in ten (10) Ghanaian endemic communities by Osei-Atweneboana *et al*. (2012) to analyze the response of the beta-

tubulin gene of *O. volvulus* to ivermectin by analyzing worm DNA for association between genotype and parasitological phenotypic response. Embryogramme analysis indicated poor response worms from subjects responding poorly to ivermectin treatment had a significantly higher reproductive activity than good response worms from subjects responding well to ivermectin treatment. An analysis of the phenotypic and genotypic response of *O. volvulus* to ivermectin treatment showed that the genotype (1183GG/1188CC/1308TT/1545GG) has been selected and showed strong association with the resistance phenotype.

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Study design

The study was a longitudinal one. It was geared towards analyzing the phenotypic as well as genotypic changes in the worms from ivermectin treated subjects. Female worms were excised from the treated subjects from June 2004 to October 2006. Samples taken were preserved in absolute ethanol and stored in a fridge at a temperature of -4°C from June 2004 to October 2006. Forty (40) female worms were analyzed at the Molecular Laboratory of the Council for Scientific and Industrial Research (CSIR) in Accra.

3.2 Study area and population

The worms that were archived were collected from Asubende in the Pru district, Kyingakrom in the Tain district and Agborlekame in the Bole district (the Pru and Tain districts are located in the Brong Ahafo region and the Bole district is also located in the Northern region). The Pru district lies between latitudes 7^o50 N and 8^o 22′ N and longitudes 0^o 30′ W and 1^o 26′ W with a land area of 2,195 sq. km. The primary occupations of these people are mainly farming and fishing. The Bole district lies within 9° 2′ N and 8° 45′ N, and longitudes 2° 29′ W and 1° 47′ W with a land surface area of 4,800 sq. km. The main occupation of these people is farming. The Tain district is located within latitudes 7° 5′ N and 8° 45′ N and longitudes 2° 52′ W and 0° 28′ E. It has a land surface area of 4,125 sq. km. The Tain River Basin and parts of the Black Volta River Basin are located within the Tain district. The primary occupation of the inhabitants is farming.

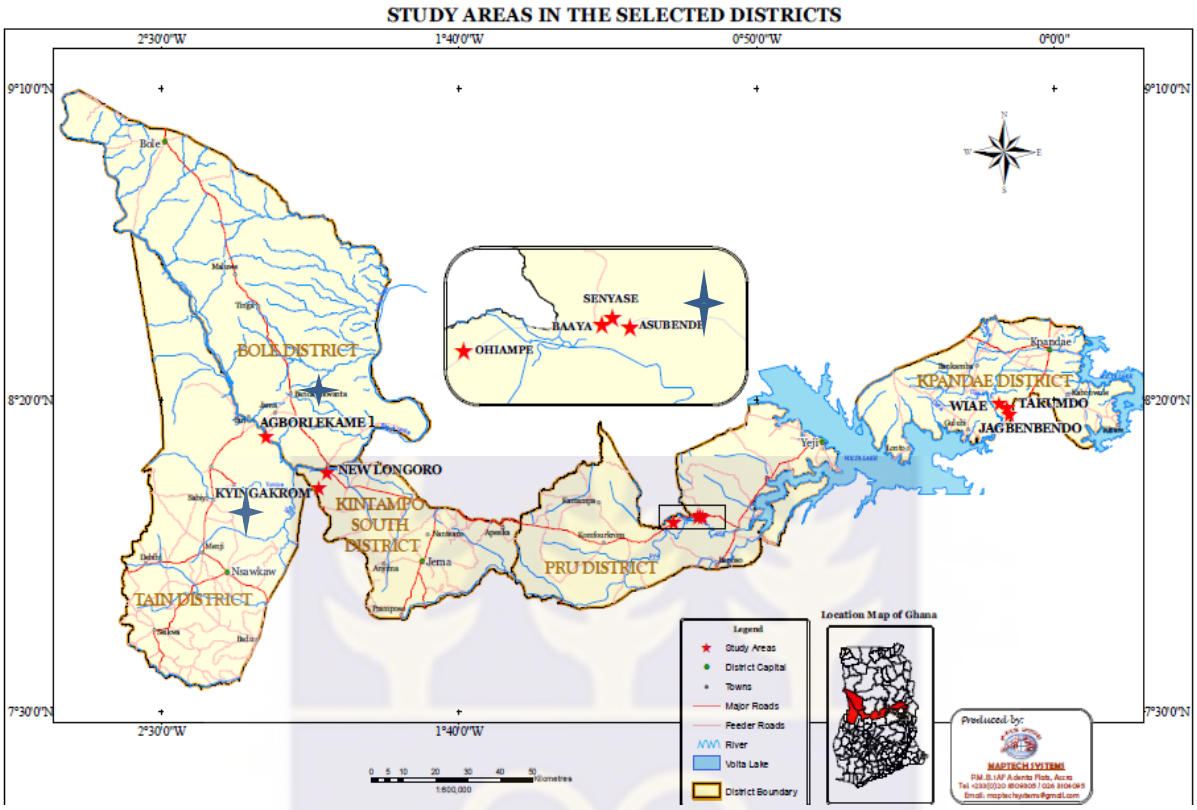

Fig.3.1: A map of the study sites (represented by symbol ✦); Source: Map Tech Systems

3.3 Inclusion criteria

The worms were obtained from subjects whose ages were between 18 and 65 years. These were people who had previously undergone at least 16 rounds of (150 µg/kg) ivermectin treatment and consented written to partake in the study.

3.4 Ethical issues

Approval for the study was sought from the Ethical and Protocol Review Committee of the College of Health Sciences, University of Ghana prior to the commencement of the study. Please, find attached the certificate of approval from the committee (appendix 1).

3.5 Nodule digestion and Isolation of adult *Onchocerca volvulus*

The nodules were preserved in liquid nitrogen and transported from the field to the CSIR. All unwanted tissues were removed from the nodules. The nodules were then placed in 50 ml tubes which contained 10 ml of 0.5% collagenase in sterile medium 199 solution. They were digested at 37^{o}C in a shaking waterbath for 10–24 hours. The worms were washed clean with normal saline. Using a dissecting microscope, live and dead worms at the time of nodulectomy were determined (Live worms were intact in morphology whiles dead worms were not). Intact female worms were stored in absolute ethanol before further work was done on them.

3.6 Categorization of *Onchocerca volvulus* based on response to ivermectin treatment

Female worms were categorized as good or poor response worms. Good response female worms were characterized by the presence of more degenerate or abnormal MF found in the genital tracts obtained from subjects who responded well to ivermectin treatment for over ten years without repopulation whilst poor response worms were those characterized by the presence of more normal stretched MF obtained from subjects who responded poorly to the treatment.

3.7 Embryogramme

Out of the forty (40) female worms assessed by the embryogramme technique, 18 were from Asubende, 10 were from Kyinakrom and 12 were from Agborlakeme.

One (1) to two (2) cm of the anterior and posterior portions of each female worm was cut. The rest of the worm was homogenized in 1 ml of saline in sterile plastic mortar. The uterine contents of the worm were squeezed out with a plastic pestle. Aliquots of the homogenates

were charged with a counting chamber. The viability of the intra-uterine MF was determined and enumerated under a light microscope. Intact stretched MF were considered as normal or alive whiles degenerated or beaded MF were considered as abnormal or dead at the time of nodulectomy.

Using a calibrated pipette, 10µl of intra-uterine MF of each female worm was pipetted onto a ring slide. Using a 10X magnification of an inverted microscope, each sample was scanned for abnormal and normal MF. Using a 40X magnification to observe, five groups of pooled MF from each sample was pipetted into PCR tubes containing 2µl of distilled water. The MF were cautiously pipetted ensuring the absence of any other tissues.

3.8 Extraction of the deoxyribonucleic acid (DNA) from beta-tubulin gene

The cut ends of the female worms that were collected were separately placed in labeled 1.5 ml eppendorf tubes and macerated with a sterile plastic pestle. DNA was extracted from female worms using a slightly modified protocol of chelex DNA extraction by Walsh *et al*. (1991). Eighty (80 µl) of 10% chelex solution and 1.5 µl of proteinase K was added to degrade proteins. The samples were incubated at 60^{o}C for an hour and mixed thoroughly by vortexing for 15 to 30 sec (to ensure thorough mixing) and centrifuged at 8,000 rpm for one (1) min. The mixture was incubated at a temperature of 95^{o}C for 45 mins. Vortexing was repeated for 30 sec and centrifuged at 10,000 rpm for 1 to 2 mins. The supernatant which contained the DNA was pipetted into labeled new 1.5 µl eppendorf tubes. For the pooled MF, 19.7µl of PCR direct and 0.3µl of proteinase K was added to degrade the proteins. The mixture was incubated at a temperature of 65°C overnight, 55°C for sixteen hours and deactivation at 85°C for 45 mins.

3.9 Amplification

The region 538bp of the beta-tubulin gene was amplified with the set of primers: Ov IVM Tub-F2 (5^1-GAGATGGATAATATGGACTAG-3^1) and Ov IVM Tub-R2 (5^1-GATCCACCAAATTGCACCTG-3^1). The fragment 538 bp of the beta-tubulin was amplified in a PCR machine for each sample. The gene was amplified in a 15 µl master mix containing 7.5 µl of syber mix, 0.2 µl each of the forward and reverse primers, 2.1 µl of ddH_2O, and 5 µl of DNA. The PCR mix was subjected to 45 amplification cycles, with each cycle consisting of 30 secs denaturation at 94°C, 45 sec annealing at 54°C, and 1 min final extension at 72°C. Holding was done at 22 °C for the final PCR products before storing at -20°C.

3.10 Agarose gel electrophoresis

Agarose gel (1.5%) was prepared and stained with 5 µl of 1X ethydium bromide. Seven (7 µl) of each PCR products was mixed with 3 µl of 1X DNA loading dye. A 50 base pair DNA ladder (New England Biolabs Inc.) was ran on all gels. Gel electrophoresis was done at 80 V and ran for an hour. After electrophoresis, the gel was visualized on a bench top Ultra-violet trans-illuminator.

3.11 Restriction Fragment Length Polymorphism (RFLP)

The restriction enzyme Mph1103I was used to digest the PCR products. The reactions for the restriction were incubated at 37°C for 2 hrs and then analyzed on a 2% agarose gel, 1X TAE,

electrophoresis at 100 V for 1 hr. The gels were stained with ethydium bromide (5 µl), and visualized with Ultra-violet trans-illuminator.

3.12 Statistical analysis

The effect of ivermectin was based on the viability of the MF, that is whether dead or alive MF (binary variable). Similarly, the phenotypic outcome was classified as Normal MF and Abnormal MF. The chi-square test was used to determine significant differences in poor and good response worms. Significant level was set at $\alpha = 0.05$.

CHAPTER FOUR

4.0 RESULTS

4.1 Embryogramme

Poor response female worms were more compared to good response female worms as shown below (Table 4.1). P- Value= 0.01.

Names of Communities	**Number of subjects**	**Number of good response female worms (%)**	**Number of poor response female Worms (%)**	**Total number of worms**
Asubende	3	4(22%)	14(78%)	18
Kyingakrom	5	4(40%)	6(60%)	10
Agborlekame	8	4(33%)	8(67%)	12
Total	16	12(30%)	28(70%)	40

Table 4.1: Distribution of good and poor response female worms from subjects in three communities

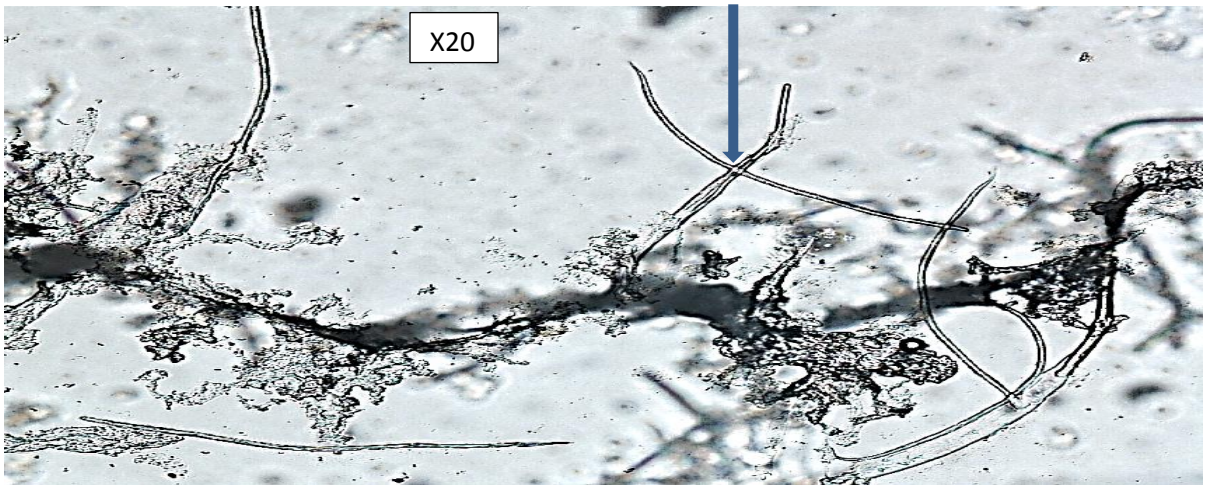

Figure 4.1: Stretched normal microfilariae (arrow) from a poor ivermectin responding subject

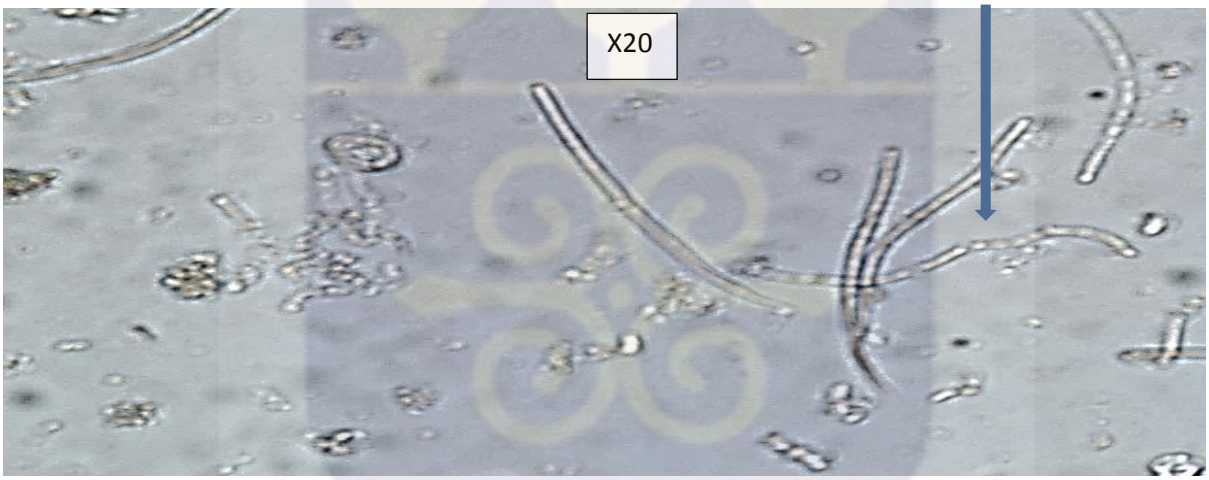

Figure 4.2: Abnormal microfilariae (arrow) from a good ivermectin responding subject

4.2 Amplification of adult female worm beta-tubulin gene

Only 14 DNA samples showed amplification on the 1.5% agarose gel as shown below (Fig. 4.3). The gel showed no amplification bands for negative template control (NTC) indicating that the various samples were not contaminated.

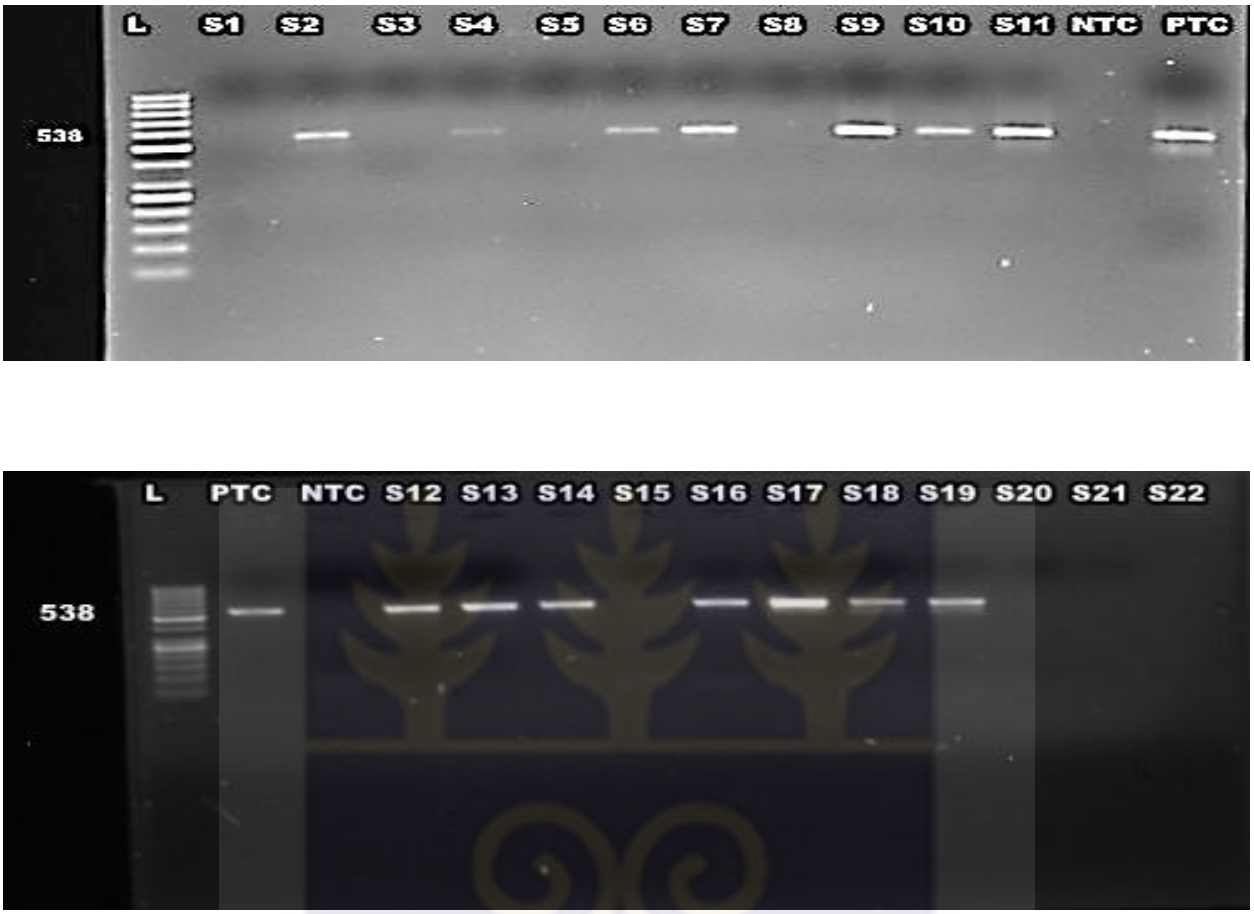

Fig. 4.3: Image of agarose gels showing adult DNA bands of beta- tubulin gene amplicons

Key:

L: 50 bp DNA marker; S2, S4, S6, S7, S9, S10, S11, S13, S14, S16, S17, S18, and S19: DNA samples with amplified gel bands; NTC: Negative template control sample of gel; S1, S3, S5 and S8, S15, S20, S21, and S22: DNA samples with no positive amplification gel bands

4.3 PCR-RFLP for adult female worms

Out of the fourteen (14) amplified samples from adult female worms which were restricted, only nine (9) gave successful bands. The result showed one (1) homozygote wild type and eight (8) heterozygote mutants as shown below (Fig. 4.4).

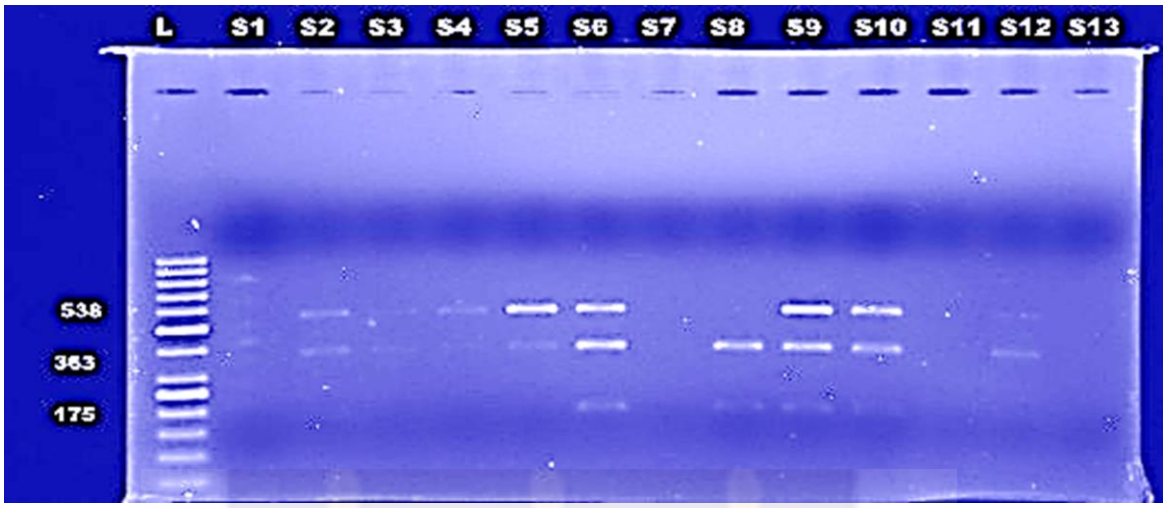

Fig.4.4: Agarose gel showing restriction bands for adult female worms

Homozygote wild type: S8; Heterozygote mutants: S2, S3, S4, S5, S6, S7, S9, S10, S12

S1, S11 and S13: Samples with no positive restriction gel bands.

4.4 Amplification of microfilariae beta-tubulin gene

Of the fourteen (14) worms that amplified, six (6) had stretched normal MF (poor response) while the remaining eight (8) had abnormal MF (good response). No statistical difference ($p>0.05$) was found between these figures**.** Only fourteen (14) out of the 24 groups of the pooled MF samples from 6 poor response worms gave amplification bands on the 1.5% agarose gel. The gels showed no amplification bands for the negative template control (NTC) (Fig.4.5).

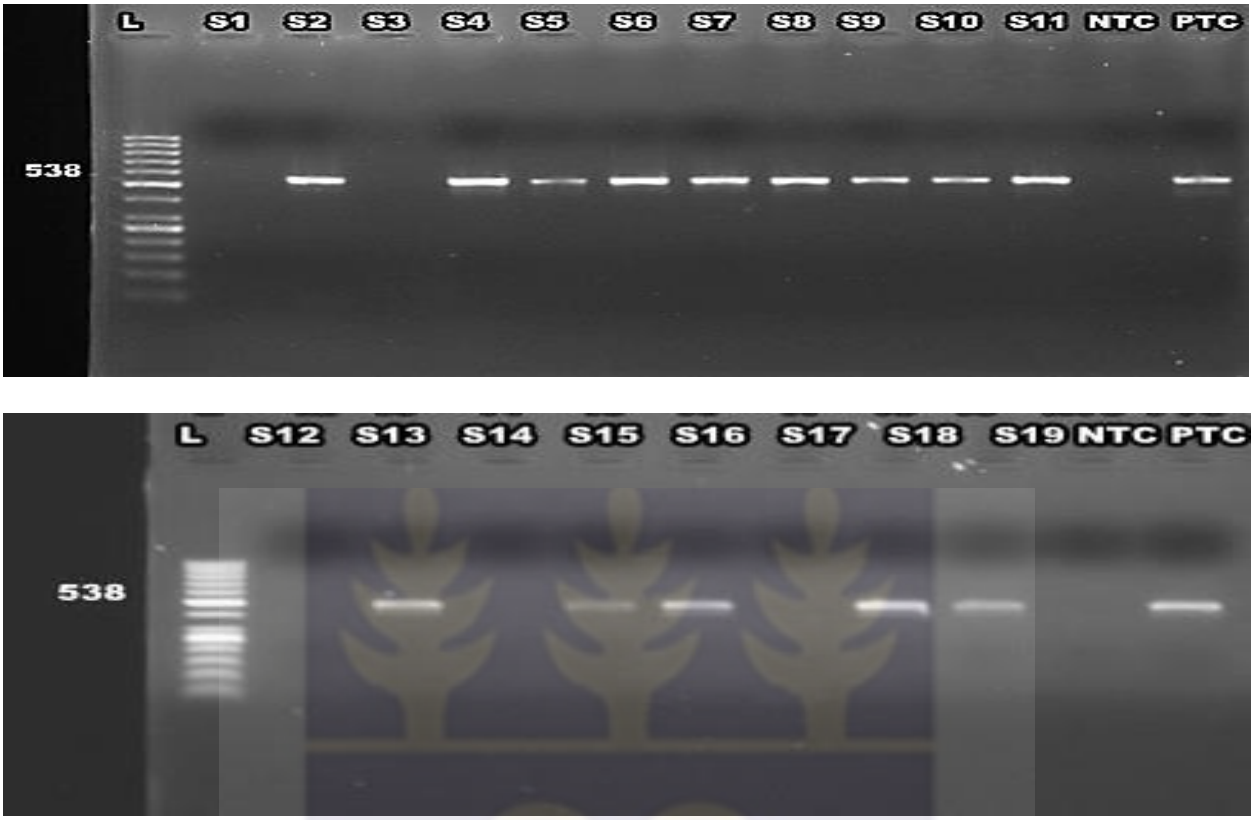

Fig. 4.5: Image of agarose gels showing microfilariae DNA bands of beta-tubulin gene amplicons.

Key: L: 50 bp DNA marker; S1, S3, S12, S14 and S17: DNA samples with no positive amplification gel bands; S2, S4-S11, S13, S15, S16, S18 and S19: DNA samples with amplified gel bands; NTC: Negative template control sample of gel; PTC: Positive template control sample of gel

4.5 PCR-RFLP for microfilariae

Only eleven (11) groups of pooled MF restricted. The RFLP result showed sample 1 was a homozygote wild type whiles samples 2 to 11 were heterozygote mutants and sample 12 and 13 did not show any bands as shown below (Fig.4.6).

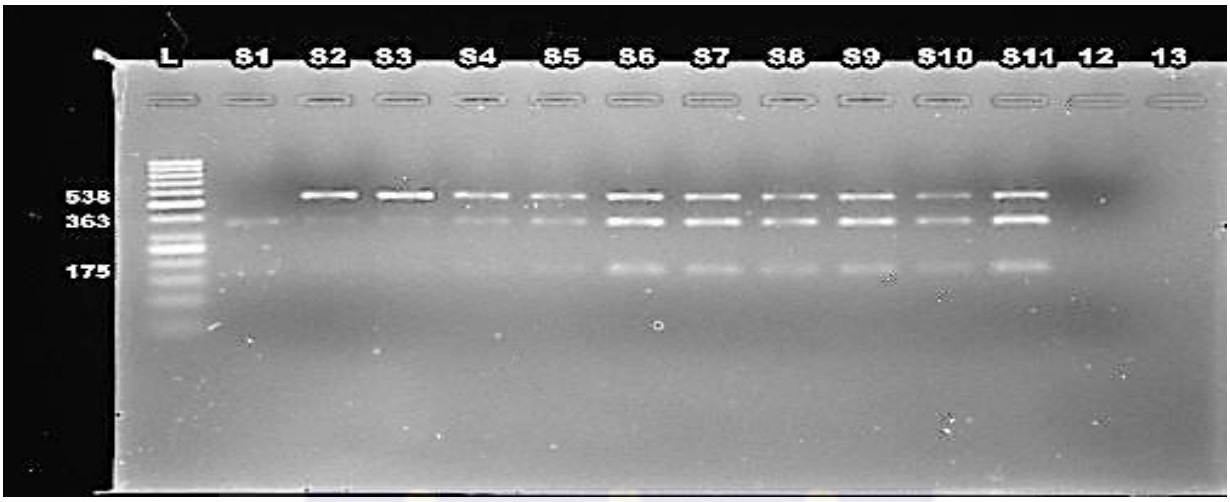

Fig. 4.6: Image of agarose gel showing restriction bands for microfilariae

Homozygote wild type: S1; Heterozygote mutant: S2, S3, S4, S5, S6, S7, S8, S9, S10, S11

Samples with no positive restriction gel bands: S12 and S13

CHAPTER FIVE

5.0 DISCUSSION

5.1 Embryogramme

Majority of the worms belonged to the group of poor response worms from patients responding poorly to ivermectin treatment. This observation implies that some of the adult female worms population are not responding to the anti-fecundity effect of ivermectin treatment. This finding is similar to those found in poor response subjects in other studies (Awadzi *et al*., 2004; Awadzi *et al.*, 2012). Studies show that poor response subjects to ivermectin treatment are subjects who are not responding well to the treatment and have worms undergoing active embryogenesis despite more than 10 annual standard doses of ivermectin treatment (Huges *et al*., 2014). Findings from this study show that the poor response female worms have stretched normal MF in their genital tract. Furthermore, this study implies ivermectin SOR has been selected for in *O. volvulus* which conforms to findings of Awadzi *et al.* (2004). Whiles these researchers found that some of the poor response worms were mostly characterized by abnormal MF; this study result shows that poor response worms were mostly characterized by stretched normal MF. Also, this recent study finding indicates that some populations of the female worms are responding to the anti-fecundity effects of ivermectin treatment which implies ivermectin kills the stretched MF. This finding is similar to those of Huges *et al*. (2014) who found abnormal MF in the genital tract of good response female worms from patients who received repeated doses of ivermectin treatment.

5.2 Genetic changes in beta-tubulin and SOR

In general, all the poor response worms which amplified in this study had SNPs. This observation implies that there are genetic changes in the beta-tubulin of the poor response female worms. This finding is similar to those found in other studies (Eng & Prichard, 2005; Eng *et al*., 2006; Bourguinat *et al.*, 2007). Studies show that poor response adult female worms from SOR patients have SNPs in their beta-tubulin gene (Huges *et al*., 2012; Osei-Atweneboana *et al*., 2012). Findings from this study showed the poor response worms have SNPs (Fig. 4.4) in their beta-tubulin gene. Also, results from this study shows that ivermectin SOR has been selected for in *O. volvulus* with SNPs in the heterozygote genotype which is in agreement with findings from Huges *et al*. (2012) and Osei-Atweneboana *et al*. (2012) studies. While these researchers found strong selection at 4 and 6 SNP positions, results from this study shows SNPs occurred at one position because the restriction enzyme digested the DNA at only one position. In addition, their findings indicated strong association of the genotypes at all four SNP positions with the resistance phenotypes. On the contrary, this study finding showed that poor response phenotype worms were strongly associated with genotypes at a single SNP position. There was association ($p<0.05$) between genetic changes in the beta-tubulin of heterozygote mutant female worms and poor response worms from patients who responded poorly to ivermectin treatment.

The result from this study is consistent with those of Eng *et al*. (2006) which showed that ivermectin has been selected for and causes changes in the alleles of the beta-tubulin gene of *O. volvulus* and *H. contortus*. Likewise, this study finding agree with those of Bourguinat *et al*. (2007) who also found that ivermectin selects for beta-tubulin in heterozygotes female worms.

5.3 Genetic changes in beta-tubulin of adult worms and microfilariae

Single nucleotide polymorphisms were found in poor response worms and their corresponding intra-uterine MF. One of the factors that affect genetic variation is mutation. These include the replacement of one DNA base pair for another, the deletion or insertion of one or more base pairs as well as changes in the chromosomes such as inversions and translocation. For genetic changes in the beta-tubulin of adult worms and their corresponding MF, findings from the present study indicate that adult female worms have passed on the mutations to their progenies. This study result clearly indicates genetic selection has occurred in *O. volvulus*.

5.4 Conclusion

Despite several rounds of ivermectin treatment, MF still survived in the genital tracts of the adult female worms even after further treatment. Genetic changes in the beta-tubulin of the female *O. volvulus* are associated with SOR to ivermectin treatment. It was also detected that some adult worms have passed some mutations on to their progenies.

5.5 Recommendations

1. Molecular markers should be incorporated in monitoring resistance in subsequent onchocerciasis studies.

2. More work in this area should be done by way of increasing the sample size and extending the work to other areas of Ghana in order to confirm the findings from this work and previous work.

3. Patients who are responding poorly to ivermectin treatment should be treated with doxycycline to avoid the spread of the resistance gene in the population.

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APPENDICES

Appendix 1

Embryogramme result

Communities	Number of subjects	Number of Adult worms	Microfilariae enumerated		
			Total	Percentage normal	Percentage abnormal
Asubende	3	18	5,228,000	4	96

Kyingakrom	5	10	748,015	60	40
Agborlakame	8	12	532,000	7	93
Total	**16**	**40**	**6,508,015**	**8.53**	**91.47**

Appendix 2

Certificate of approval

UNIVERSITY OF GHANA
COLLEGE OF HEALTH SCIENCES
ETHICAL AND PROTOCOL REVIEW COMMITTEE

My Ref. No.....

30th June, 2016.

Ms. Judith Esinam Yabani
Department of Medical Microbiology
School of Biomedical and Allied Health Sciences
University of Ghana
Korle-Bu, Accra

Dear Ms. Yabani,

ETHICAL CLEARANCE

Protocol Identification Number: **CHS-Et/M.8 – P 3.3/2015-2016**

The Ethical and Protocol Review Committee of the College of Health Sciences on the 29th of June, 2016 unanimously approved your research proposal.

TITLE OF PROTOCOL: **"Phenotypic and Genotypuic analysis of *Onchocerca volvulus* response to Ivermectin"**

PRINCIPAL INVESTIGATOR: **Ms. Judith Esinam Yabani**

This approval requires that you submit six-monthly review reports of the protocol to the Committee and a final full review to the Ethical and Protocol Review Committee at the completion of the study. The Committee may observe, or cause to be observed, procedures and records of the study during and after implementation.

Please note that any significant modification of this project must be submitted to the Committee for review and approval before its implementation.

You are required to report all serious adverse events related to this study to the Ethical and Protocol Review Committee within seven (7) days verbally and fourteen (14) days in writing.

As part of the review process, it is the Committee's duty to review the ethical aspects of any manuscript that may be produced from this study. You will therefore be required to furnish the Committee with any manuscript for publication.

This ethical clearance is valid till 31st March, 2017.

Please always quote the protocol identification number in all future correspondence in relation to this protocol.

Signed: .....
PROFESSOR ANDREW A. ADJEI
CHAIRPERSON, ETHICAL AND PROTOCOL REVIEW COMMITTEE

cc: Provost, CHS
Dean, SBAHS
Head of Department

P. O. Box 52, Korle-bu, Accra, Ghana • Tel: +233 (0) 302665103/244061270 • Fax: +233 (0) 302660762
• Email: eprc@chs.edu.gh / provost@chs.edu.gh • Website: www.chs.ug.edu.gh